The EMBO Journal Vol. 20 No. 22 pp. 6550–6558, 2001

Non-homologous end-joining proteins are required for *Agrobacterium* T-DNA integration

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Agrobacterium tumefaciens **causes crown gall disease in dicotyledonous plants by introducing a segment of DNA (T-DNA), derived from its tumour-inducing (Ti) plasmid, into plant cells at infection sites. Besides these natural hosts,** ***Agrobacterium*** **can deliver the T-DNA also to monocotyledonous plants, yeasts and fungi. The T-DNA integrates randomly into one of the chromosomes of the eukaryotic host by an unknown process. Here, we have used the yeast** ***Saccharomyces cerevisiae*** **as a T-DNA recipient to demonstrate that the non-homologous end-joining (NHEJ) proteins Yku70, Rad50, Mre11, Xrs2, Lig4 and Sir4 are required for the integration of T-DNA into the host genome. We discovered a minor pathway for T-DNA integration at the telomeric regions, which is still operational in the absence of Rad50, Mre11 or Xrs2, but not in the absence of Yku70. T-DNA integration at the telomeric regions in the** ***rad50***, ***mre11*** **and** ***xrs2*** **mutants was accompanied by gross chromosomal rearrangements.**

Keywords: *Agrobacterium*/genomic instability/non-homologous end-joining /T-DNA integration/telomeres

Introduction

Agrobacterium tumefaciens causes crown gall disease in plants by transferring an oncogenic segment of DNA, the transferred or T-DNA, to plant cells at wound sites (Chilton *et al*., 1977; Tinland and Hohn, 1995). The T-DNA is derived from an ~200 kb tumour-inducing (Ti) plasmid, which is present in the bacterium. Plant phenolic compounds, produced by the wounded plant cells, induce expression of virulence genes located elsewhere on the Ti plasmid. The virulence protein VirD2 introduces nicks at the 24 bp border repeats, which flank the T-region (Wang *et al*., 1987; Pansegrau, 1993). This leads to the formation of a single-stranded DNA copy from the T-region, which is called the T-strand (Stachel *et al*., 1986). The T-strand is bound at the 5′ end by the VirD2 protein and it is this single stranded nucleoprotein complex that is delivered into plant cells (Ward and Barnes, 1988; Chaudhury *et al*., 1994; Tinland *et al*., 1994; Yusibov *et al*., 1994). There it is co-operatively bound by the VirE2 protein (Citovsky *et al*., 1989), which is delivered separately into plant cells by the bacterium (Vergunst *et al*., 2000), and targeted to the nucleus by the presence of a nuclear localization signal in the VirE2 and VirD2 proteins (Tinland *et al*., 1992; Rossi *et al*., 1993; Tzfira *et al*., 2000). T-DNA integration occurs at random positions in the genome by a process of non-homologous recombination (NHR) (Offringa *et al*., 1990; Mayerhofer *et al*., 1991; Tinland and Hohn, 1995; Gelvin, 2000). Although the processing and transfer of T-DNA to plants is reasonably well understood, (host) factors involved in T-DNA integration are just beginning to be identified (Gelvin, 2000; Mysore *et al*., 2000).

The T-DNA itself does not encode enzymes that are involved in integration. As the *Agrobacterium* proteins VirD2 and VirE2 accompany the T-DNA to the plant nucleus, it is reasonable to propose that they may be involved in T-DNA integration. The VirD2 protein indeed is important for the accuracy of the integration of T-DNA (Tinland *et al*., 1995). The VirE2 protein probably protects the T-DNA from nucleolytic degradation and eases its translocation into the nucleus (Rossi *et al*., 1996; Zupan *et al*., 1996). Therefore, VirD2 and VirE2 are important for T-DNA transfer and nuclear targeting, but do not seem to play an essential role in the integration process *per se*. In accordance with this, *in vitro* studies showed that a T-DNA ligation-integration reaction is mediated by plant enzymes, which implies a role for host factors in T-DNA integration (Ziemienovicz *et al*., 2000).

T-DNA transfer can also occur, at least under laboratory conditions, to yeasts and fungi (Bundock *et al*., 1995; Bundock and Hooykaas, 1996; De Groot *et al*., 1998; Gouka *et al*., 1999) where, in the absence of DNA homology, integration occurs by a similar process of NHR as in plants. In contrast, integration occurs by homologous recombination (HR) when the T-DNA carries homology with the yeast genome. This was not found in plants where T-DNA sharing extensive homology with the plant genome still integrates mainly by NHR (Offringa *et al*., 1990). These important findings indicate that the process of T-DNA integration into the host genome is predominantly determined by host factors. Recently, Salomon and Puchta (1998) showed that T-DNA could be captured during DNA double-strand break (DSB) repair in plants. This suggests that DSB repair provides a pathway for T-DNA integration. However, as the right ends of the T-DNAs that had integrated into the DSB were all truncated, it is possible that this does not represent the most common form of T-DNA integration.

Studies on the repair of DNA DSBs in the yeast *Saccharomyces cerevisiae* revealed that there are two general recombination mechanisms: one that requires homology between the two recombining DNA molecules (HR) and one that is independent of such homology [non-homologous end-joining (NHEJ)] (reviewed by Critchlow and Jackson, 1998; Haber, 2000). Several mechanisms have been described for the repair of DSBs by HR, most of

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which rely on the action of genes of the *RAD52* epistasis group (*RAD50-RAD59*, *MRE11* and *XRS2*) (reviewed by Sung *et al*., 2000). Studies on the repair of DSBs under conditions where HR was impossible revealed that at least 10 genes are required for repair by NHEJ (*YKU70*, *YKU80*, *LIG4*, *LIF1*, *SIR2*, *SIR3*, *SIR4*, *RAD50*, *MRE11* and *XRS2*) (reviewed by Tsukamoto and Ikeda, 1998; Lewis and Resnick, 2000). Most of these NHEJ genes have additional functions in telomere length maintenance (*RAD50*, *MRE11*, *XRS*, *YKU70*, *YKU80* and *SIR2-SIR4*; Porter *et al*., 1996; Boulton and Jackson, 1998; Chamankkah and Xiao, 1999; Gallego and White, 2001) and/or transcriptional silencing at the telomeres (*YKU70*, *YKU80* and *SIR2-SIR4*; Aparicio *et al*., 1991; Boulton and Jackson, 1998).

We have now studied the role of host proteins in the integration of T-DNA by NHR. As the results obtained so far on this topic with plants are controversial (Gelvin, 2000), we have now employed the yeast *S.cerevisiae* as a model organism to investigate which of the genes encoding for recombination enzymes are necessary for T-DNA integration. To this end T-DNA integration in wild-type was compared with that in isogenic strains carrying disruptions of these genes. The results show for the first time that the NHEJ proteins Yku70, Rad50, Mre11, Xrs2, Lig4 and Sir4 are required for the integration of T-DNA into the host genome. We discovered a minor pathway for T-DNA integration at the telomeric regions, which is still operational in the absence of Rad50, Mre11 and Xrs2, but not in the absence of Yku70. T-DNA integration at the telomeric regions in the *rad50*, *mre11* and *xrs2* mutants was accompanied by gross chromosomal rearrangements.

Results

A versatile T-DNA to study integration by NHR in yeast

T-DNA, which lacks homology with the yeast genome, has been described to integrate by NHR (Bundock and Hooykaas, 1996). The T-DNA that was used in this study carried the *URA3* gene and to prevent homology between the T-DNA and the yeast genome the *URA3* gene was removed from the genome of the T-DNA recipient. In order to be able to study the integration of T-DNA by NHR in various yeast strains, independent of the genetic background, a novel T-DNA vector (pSDM8000) was constructed with the *KanMX* selectable marker between the T-DNA border repeats (Figure 1; Wach *et al*., 1994). This marker, which allows selection of transgenic yeasts resistant to G418, consists of heterologous DNA and thus the T-DNA of pSDM8000 lacks any homology with the yeast genome. Integration of the T-DNA from pSDM8000 into the yeast genome can therefore only occur by NHR. The T-DNA from pSDM8000 integrated in the wild-type yeast strains YPH250 and JKM115 at frequencies of 1.6×10^{-7} and 1.2×10^{-5}, respectively (Table I). For 10 T-DNA insertions the integration site was established after retrieval of the linked genomic sequences by the Vectorette PCR (data not shown). This confirmed that integration of the T-DNA from pSDM8000 had occurred by NHR as was described previously (Bundock and Hooykaas, 1996).

NHEJ proteins are required for T-DNA integration by NHR

Many proteins involved in various forms of DNA recombination and DNA repair have been identified in yeast. In order to determine their possible role in T-DNA integration, we performed T-DNA transfer experiments and compared T-DNA integration in wild-type yeasts with that in isogenic strains carrying disruptions of the

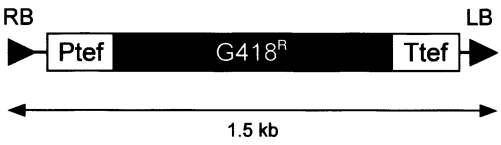


Fig. 1. Schematic representation of the T-DNA from pSDM8000. The T-DNA from pSDM8000 was used in co-cultivation experiments to study T-DNA integration by NHR in recombination defective *S.cerevisiae* strains. The T-DNA contains the *KanMX* cassette, which consists of the *kan* resistance gene of the *Escherichia coli* transposon *Tn*903 under control of transcriptional and translational sequences of the filamentous fungus *Ashbya gossypii TEF* gene. This module allows selection of *S.cerevisiae* transformants resistant against the antibiotic G418 (Wach *et al*., 1994).

Table I. Frequencies of T-DNA integration by NHR in recombination defective yeast strains

Strain	Genotype	Frequency of G418-resistant colonies ± SEM ($\times 10^{-8}$)[a]	Relative frequency of G418-resistant colonies (%)[b]
YPH250	WT	16 ± 9.6	100
YPH250*rad51*	*rad51*Δ	14 ± 7.8	88
YPH250*rad52*	*rad52*Δ	38 ± 8.4[c]	238
YPH250*yku70*	*yku70*Δ	<0.0075[c]	<0.05
YPH250*rad50*	*rad50*Δ	0.80 ± 0.40[c]	5.0
YPH250*lig4*	*lig4*Δ	0.37 ± 0.36[c]	2.3
JKM115	WT	1150 ± 0.50	100
JKM129	*xrs2*Δ	27 ± 6.0[c]	2.3
JKM138	*mre11*Δ	29 ± 3.0[c]	2.5
JKM120*sir4*	*sir4*Δ	15 ± 1.9[c]	1.3

[a]All yeast strains were co-cultivated with LBA1119(pSDM8000). Averages of two or more independent experiments are shown. Frequencies are depicted as the number of G418-resistant colonies divided by the output number of yeast cells (cells/ml). SEM= standard error of the mean.
[b]The relative frequency of T-DNA integration by NHR is (frequency in the mutant/frequency in the wild-type (WT)) × 100%.
[c]The means of the frequency of G418-resistant colonies seen in the wild-type (WT) and the mutant were tested significantly different in a Student's *t*-test ($p < 0.05$).

recombination genes *RAD51*, *RAD52*, *YKU70*, *LIG4*, *MRE11*, *RAD50*, *XRS2* and *SIR4*. The results are summarized in Table I. In the *rad51* and *rad52* mutants that are defective in HR, the frequencies of T-DNA integration by NHR were, respectively, similar and slightly higher (~2- to 3-fold) than observed for the wild-type yeast strain. The Rad51 and Rad52 proteins apparently do not play a role in T-DNA integration by NHR and possibly Rad52 even suppresses T-DNA integration by NHR. In contrast, in the *lig4*, *mre11*, *rad50*, *xrs2* and *sir4* mutants the frequency of T-DNA integration by NHR was reduced dramatically and T-DNA integration seems to be abolished in the *yku70* mutant, as no G418-resistant colonies were obtained in experiments performed with this mutant. The genes impaired in these mutants have been described as being involved in the repair of genomic DSBs by NHEJ (Tsukamoto and Ikeda, 1998; Lewis and Resnick, 2000). We conclude that the proteins Yku70, Lig4, Mre11, Rad50, Xrs2 and Sir4, are also required for the integration of T-DNA by NHR into the host genome. In addition, Yku70 seems to be essential for T-DNA integration as in its absence integration of T-DNA was never observed.

T-DNA integrates preferentially at (sub)telomeric regions in rad50, mre11 and xrs2 mutants and forms repeat structures in the genome of a lig4 mutant

From co-cultivation experiments with the *rad50*, *mre11*, *xrs2*, *lig4* and *sir4* mutants, we obtained a small number of G418-resistant colonies. The features of T-DNA integration were determined for a number of these lines. The yeast genomic sequences linked to the T-DNA were isolated by Vectorette PCR or thermal asymmetric interlaced–PCR (TAIL–PCR), sequenced and used in a BLAST search in the yeast genome database to determine the T-DNA integration sites. Strikingly, analysis of the genomic DNA junctions to the left end of the T-DNA revealed that in two out of three *rad50*, four out of six *mre11* and two out of two *xrs2* mutants analysed, T-DNAs had integrated into telomeres or subtelomeric regions (Table II; Figure 2). *Saccharomyces cerevisiae* telomeres generally consist of one or more copies of Y′ and X elements followed by telomerase-generated C(1–3)A/TG(1–3) repeats (Figure 2; Zakian, 1996). In the *rad50k.1*, *rad50k.6*, *mre11k.14*, *mre11k.17* and *xrs2k.1* mutants, the left end of the T-DNA was fused to this typical telomerase-generated C(1–3)A/TG(1–3) repeat (Figure 2). Other than these telomeric insertions we found one T-DNA insertion in the long terminal repeat (LTR) of the Ty transposable element (*mre11k.5*; Table II and Figure 2) and two insertions in the rDNA region, present in chromosome XII (*mre11k.4* and *rad50k.5*; Table II and Figure 2).

We obtained only three colonies from co-cultivations with the *lig4* mutant. Unfortunately, the location of the T-DNA insertion could not be established in these three *lig4* mutants. In one mutant, the right end of the T-DNA was found to be fused to sequences of the binary vector (*lig4k.1*; Figure 3). Most likely only the left border repeat (LB) was processed during T-strand formation in *Agrobacterium*, resulting in the transfer and integration of the binary vector. In the two other *lig4* mutants a perfect fusion between the right and left end of two T-DNAs was found (*lig4k.2* and *lig4k.3*; Figure 3). This showed that two T-DNA copies had integrated at the same position in the yeast genome, resulting in the formation of a direct repeat structure. T-DNA repeat structures were only found in ~3% of T-DNA transformed wild-type (data not shown), suggesting that the absence of Lig4 favours the formation of complex T-DNA structures in the yeast genome.

Analysis of six T-DNA insertions in the *sir4* mutant revealed no preference for integration at the telomeres as found for the wild-type strain (Table II and Figure 4). T-DNA integrations in the wild-type are characterized by truncation of the T-DNA left end and the presence of microhomology between the left end of the T-DNA and the target site (Offringa *et al*., 1990; Mayerhofer *et al*., 1991; Tinland and Hohn, 1995; Bundock and Hooykaas, 1996). Similar features were seen for T-DNA integrations in the *rad50*, *mre11*, *xrs2* and *sir4* mutants (Figures 2 and 4).

We conclude that in the *rad50*, *mre11* and *xrs2* mutants the T-DNA, if integrated at all, becomes preferentially inserted in (sub)telomeric regions. Yku70 may play an important role in this novel pathway for T-DNA integration at the telomeres as in its absence no such insertions were found. In contrast, disruption of *SIR4* did affect the efficiency of T-DNA integration by NHR, but not the position of T-DNA integration. In the *lig4* mutant T-DNA integrated rarely and formed repeat structures at so far unknown positions in the genome.

T-DNA integration in rad50, mre11, xrs2 and lig4 mutants is accompanied by chromosomal instability

Telomeres consist of highly conserved and homologous sequences (Figure 2; Zakian, 1996). When the telomere sequences flanking the T-DNA inserts in the NHEJ mutants were used in a BLAST search, several yeast chromosomes were found to be the possible target for T-DNA integration. In order to determine in which chromosome the T-DNAs had integrated in the *rad50*, *mre11*, *xrs2* and *lig4* mutants, intact chromosomes were isolated, separated in a CHEF gel, blotted on a membrane and hybridized with the KanMX probe which anneals to the T-DNA (Figure 5).

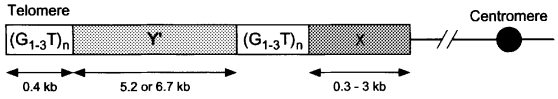
As a control, two *sir4* strains and the two *rad50* and *mre11* strains in which the T-DNA had inserted outside the telomeres were analysed. In the *sir4k.1* and *sir4k.9* mutants the T-DNA was present in chromosome XI and XV, respectively (Table II; Figure 5), confirming the sequence data. For the *rad50k.5* and *mre11k.4* mutants the sequence data indicated that T-DNA insertions were located in the rDNA region, present in chromosome XII (Figure 5). In line with this for both mutants a band on the blot corresponding to chromosome XII was seen, indicating that the T-DNA indeed had inserted in chromosome XII. In the *mre11k.5* mutant the T-DNA insertion was present in an LTR element according to the sequence analysis (Figure 5). Unexpectedly, for this mutant three bands were seen on the blot, indicating the presence of T-DNA insertions in chromosomes XV or VII (which were not visually separated on the gel), II and XI. It is conceivable that one T-DNA had integrated in a LTR element on one of these chromosomes and was

Table II. Genomic distribution of T-DNA integrated by NHR in *rad50*, *mre11*, *xrs2* and *sir4* mutants in comparison with the wild-type after T-DNA transfer from pSDM8000

Yeast strain (genotype)	(Sub)telomeric region	LTR	rDNA	Elsewhere
*rad50*Δ	2	0	1	0
*mre11*Δ	4	1	1	0
*xrs2*Δ	2	0	0	0
*sir4*Δ	0	0	0	6
Wild-type[a]	1	2	1	50

[a]Previously, target sites for T-DNA integration in the genome of *S.cerevisiae* strain RSY12 were determined in 44 T-DNA transformed lines (Bundock and Hooykaas, 1996; Bundock, 1999). In addition, we determined the position of T-DNA integration in 10 T-DNA transformed *S.cerevisiae* YPH250 lines.

A



B

Strain	5′ CAGGATATATTCAATTGTAAATCTC--- 3′ (LB′ T-DNA)	Location	Chromosome	Altered mobility
rad50k.1	5′ *TGTGGGTGTG*(-6)ATATTCAATTGTAAATCTC--- 3′	*Tel.*	VII	VII
rad50k.5	5′ *GGGGGCATCAG*(-7)TATTCAATTGTAAATCTC--- 3′	*rDNA*	XII	-
rad50k.6	5′ *AGATGTGAGAGAGTGTGTGTGGGTGT*(-25)GAAGTC 3′	*Tel.*	XV or VII, XVI, XI	XII
mre11k.4	5′ *TCTGGTA*(-3)**GATAT**ATTCAATTGTAAATCTC--- 3′	*rDNA*	XII	-
mre11k.5	5′ *CACATATTTCTC*(-8)ATTCAATTGTAAATCTC--- 3′	*LTR*	XV, II, XI	-
mre11k.8	5′ *CGACTACTTTATATC*(-11)CAATTGTAAATCTC--- 3′	*Subtel.*	XII	XII
mre11k.11	5′ *GAAGAACCCAT*(-7)TATTCAATTGTAAATCTC--- 3′	*Subtel.*	XII	XII
mre11k.14	5′ *TGGGTGTGGGT*(-7)**TATT**CAATTGTAAATCTC--- 3′	*Tel.*	II	II
mre11k.17	5′ *TGGGTGTGGTGTGT*(-9)TCAATTGTAAATCTC--- 3′	*Tel.*	n.d.	n.d.
xrs2k.1	5′ *TGTGTGGGTGTGGGT*(-10)CAATTGTAAATCTC--- 3′	*Tel.*	IV or XII, XV or VII, II	XI
xrs2k.17	5′ *CGTCAAGGATATATT*(-1)CAATTGTAAATCTC--- 3′	*Subtel.*	III	XVI, III

Fig. 2. T-DNA integrates preferentially at (sub)telomeric regions in *rad50*, *mre11* and *xrs2* mutants. (**A**) Schematic representation of a yeast chromosome end, showing the position of the telomeric ($G_{1-3}T$) repeats, the Y′ and X elements and the centromere. Y′ and X elements are present at only a subset of all chromosomes and are often found in association with internal tracts of ($G_{1-3}T$) repeats. (**B**) Junction sequences of T-DNA left end and genomic DNA of the *S.cerevisiae rad50*, *mre11* and *xrs2* mutants. Genomic DNA sequences are shown in italics, T-DNA sequences in normal capitals. Bold sequences represent microhomology of the T-DNA left end with the integration site. Filler DNA sequences are underlined and depicted in italics. The numbers above the sequences represent the number of base pairs deleted from the T-DNA left end. *Tel.* = telomeric region; *Subtel.* = subtelomeric region; *rDNA* = ribosomal DNA region; *LTR* = long terminal repeat of Ty element; – = none of the 16 chromosomes showed an altered mobility; n.d. = not determined; LB′ = remnant of T-DNA left border repeat.

subsequently duplicated and translocated to the other chromosomes (Kim *et al*., 1998). Similarly, the chromosomes into which the T-DNA had inserted in the other *rad50*, *mre11* and *xrs2* mutants were assigned (Figure 2).

The *rad50* and *mre11* mutants, that had T-DNA insertions in rDNA or LTR elements, did not show any changes in chromosome mobility when compared with the wild-type. In contrast, in all *rad50*, *mre11* and *xrs2* mutants that contained a T-DNA inserted at the (sub)-telomeric region, changes in the mobility of one or more of the chromosomes were seen. In most of these mutants, *rad50k.1*, *mre11k.8*, *mre11k.11*, *mre11k.14*, *mre11k.17* and *xrs2k.17*, the chromosome into which the T-DNA had inserted showed an altered mobility. This was confirmed by the use of chromosome specific probes (data not shown). In this way it became apparent, for instance, that the T-DNA insertion in the *rad50k.1* mutant was present on chromosome VII, which now co-migrated with chromosome IV instead of XV (Figure 5). In a minority of the mutants, *rad50k.6* and *xrs2k.1*, an altered mobility was seen of a chromosome that did not contain a T-DNA insertion. For instance, in the *rad50k.6* mutant T-DNA insertions were found in chromosome XV or VII, XVI and XI (Figures 2 and 5). None of these chromosomes showed a change in mobility. However, with a probe specific for chromosome XII a change in the mobility of this chromosome was observed, although a T-DNA insertion was not present in this chromosome (Figure 5).

Strain	T-DNA structure	Chromosome	Altered mobility
	T-DNA RB Binary vector		
lig4k.1	5′–GGCAGGATATATACCGTTGTAATT*GTACCGAGCTCGTGTGA*--3′	XV or VII, XIII, II	-
	T-DNA RB' LB' T-DNA		
lig4k.2	5′--*GAACTCGA***GG**CAGGATATATTCAATTGTAAAT*CTCGAAGT*--3′	V, VIII	XII
	T-DNA RB' LB' T-DNA		
lig4k.3	5′--*GAACTCGA***GG**CAGGATATATTCAATTGTAAAT*CTCGAAGT*--3′	IV	-

Fig. 3. T-DNA forms repeat structures in the genome of a *lig4* mutant. Sequences of T-DNA structures as found in the genome of the *S.cerevisiae lig4* lines are depicted. Sequences of the binary vector are underlined and depicted in italics. T-DNA sequences are shown in italics, except for the border sequences. The right border repeat (RB) is presented in bold for *lig4k.2* and *lig4k.3* to distinguish between RB and LB sequences. RB′ and LB′ = fused remnants of the RB and LB, respectively.

Strain	LB' T-DNA CAGGATATATTCAATTGTAAATCTC--- 3′	Chromosome	Position
sir4k..1	0 5′ *AAAC*CAGGATATATTCAATTGTAAATCTC--- 3′	XI	171364
sir4k..2	-6 5′ *CGAGTGGGAC*ATATTCAATTGTAAATCTC--- 3′	XVI	404327
sir4k.5	0 5′ *CTAG*CAGGATATATTCAATTGTAAATCTC--- 3′	XIII	458128
sir4k..6	-3 5′ *AGAGAAA*GATATATTCAATTGTAAATCTC--- 3′	XIII	756597
sir4k.9	0 5′ *ATAG*CAGGATATATTCAATTGTAAATCTC--- 3′	XV	516899
sir4k.10	-6 5′ *ATGACGGGAAA*TATTCAATTGTAAATCTC--- 3′	XIII	837130

Fig. 4. The position of T-DNA integration by NHR in the genome of a *sir4* mutant is not biased. Junction sequences of the T-DNA left end and genomic DNA of the *sir4* mutant are depicted. Genomic DNA sequences are shown in italics, T-DNA sequences in normal capitals. The numbers above the sequences represent the number of base pairs deleted from the left T-DNA end.

The position of the T-DNA insertions in the *lig4* mutant could not be determined as sequence analysis only revealed T-DNA repeat structures. CHEF gel analysis of the *lig4k.1*, *lig4k.2* and *lig4k.3* mutants revealed that T-DNA had integrated in chromosome XV or VII, XIII and II (*lig4k.1)*, V and II (*lig4k.2*) and IV (*lig4k.3*) (Figures 3 and 5). In the *lig4k.2* mutant chromosome XII had an altered mobility, although a T-DNA had not integrated into this chromosome.

In summary, by combining sequence and CHEF-gel data we can draw several conclusions. In the NHEJ mutants *rad50*, *mre11* and *xrs2* the T-DNA integrates preferentially into telomeric regions, but these events are associated with the formation of gross chromosomal rearrangements as detected by altered chromosome mobility. Strikingly, the chromosomes that show rearrangements are not always the chromosomes that contain the T-DNA insertion. As we did not observe chromosomal rearrangements in the wild-type strain transformed by *A.tumefaciens* or in untransformed *rad50*, *mre11*, *xrs2* and *lig4* mutants (data not shown) we can only conclude that introduction and/or integration of T-DNA in these NHEJ mutants is accompanied by genetic instability. Whether this instability is a consequence of T-DNA integration at the telomeres or that T-DNA is captured at the telomeres during repair of already existing telomeric instability in the NHEJ mutants needs to be investigated.

Discussion

Agrobacterium tumefaciens delivers T-DNA into cells of its natural host, dicotyledonous plants, monocotyledonous plants, yeasts and fungi. In the absence of homology with the host genome the T-DNA integrates randomly into any of the chromosomes of these eukaryotic hosts by NHR. Here, we have used the yeast *S.cerevisiae* as a T-DNA recipient to investigate which host proteins might play a role in T-DNA integration. We demonstrate that the NHEJ proteins Yku70, Rad50, Mre11, Xrs2, Lig4 and Sir4 are required for the integration of T-DNA into the host genome. It was described before that these proteins have distinct functions in the repair of genomic DSBs by NHEJ (Tsukamoto and Ikeda, 1998; Lewis and Resnick, 2000). We infer from this that DNA DSB repair by NHEJ provides a pathway for T-DNA integration. A recent study showed that T-DNAs can indeed be captured during DSB repair in plants (Salomon and Puchta, 1998) However, as the right ends of the T-DNAs that had integrated into the DSB were all truncated, in contrast to the right ends of the most commonly found T-DNAs, it is probable that T-DNA integration occurs usually at other sites than DSBs. The present work shows that such T-DNA integrations are nevertheless dependent on NHEJ enzymes.

It would be interesting to know whether the dependence on the NHEJ enzymes for integration is typical for the *Agrobacterium* T-DNA or more general for introduced non-homologous DNA molecules. Schiestl *et al*. (1994) showed that a mutation in *RAD50* reduces the frequency of integration of non-homologous, *Bam*HI-treated DNA into the yeast genome by restriction enzyme-mediated integration (REMI) after yeast transformation. The role of *RAD50* in such integration can be explained by the fact that the accompanying restriction enzyme induces DSBs in the genome where integration can occur by NHEJ.

The Rad51 and Rad52 proteins play important roles in HR in yeast. The frequency of T-DNA integration by NHR was comparable with wild-type levels in the *rad51* mutant, but >2-fold higher in the *rad52* mutant. This latter

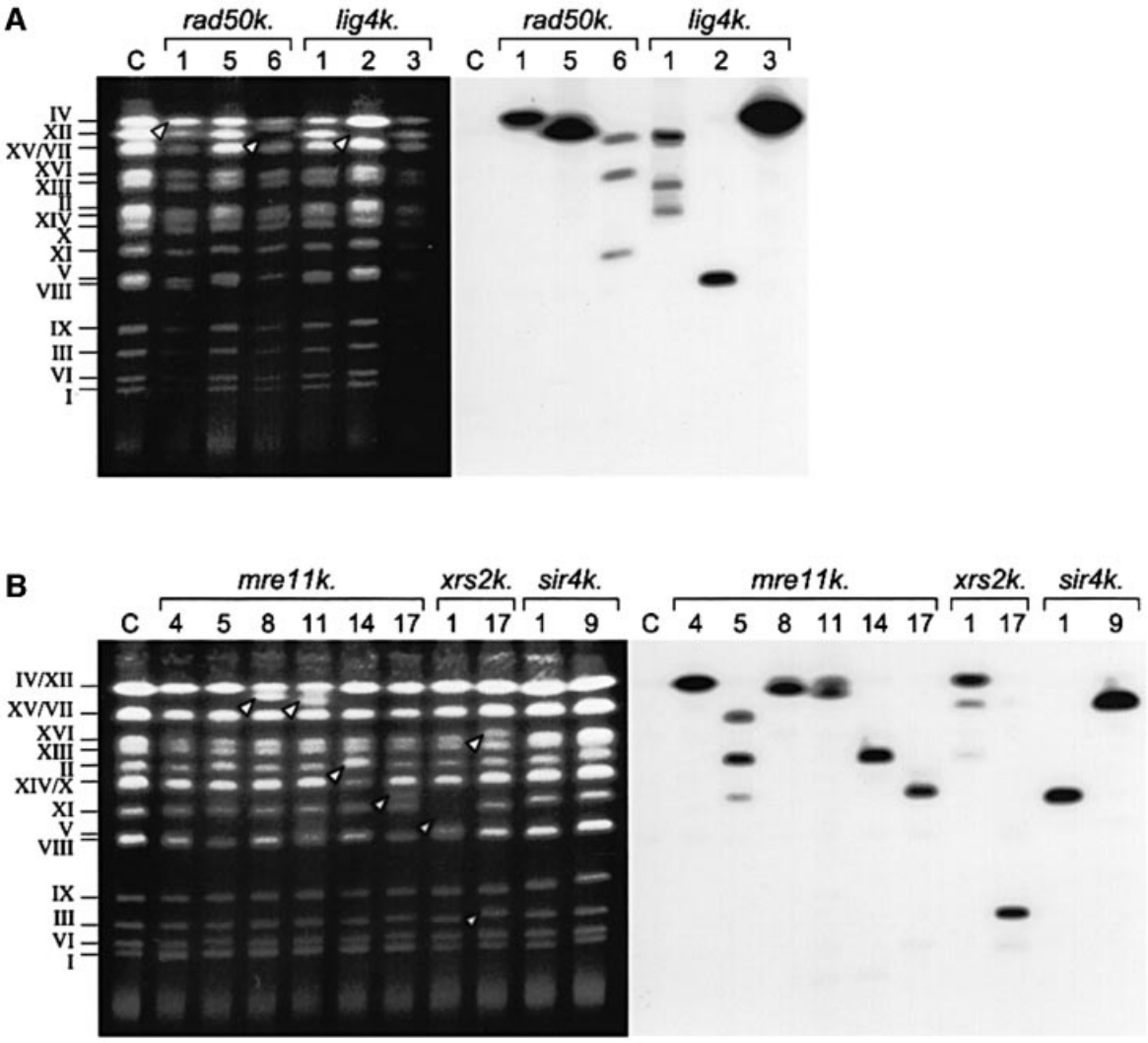


Fig. 5. T-DNA integration is accompanied by chromosomal rearrangements in *rad50*, *mre11*, *xrs2 and lig4* mutants. CHEF-gel analysis of T-DNA integration events in the genome of *S.cerevisiae rad50*, *mre11*, *xrs2*, *lig4* and *sir4* mutants was studied. Chromosomes from G418-resistant *S.cerevisiae* colonies obtained after co-cultivation with *A.tumefaciens* carrying pSDM8000 were isolated, separated on a CHEF gel (left panels in A and B) and blotted on a membrane. The membrane was hybridized with a labelled KanMX probe that anneals to the T-DNA and an autoradiograph was made (right panels in A and B). (**A**) T-DNA transformed *rad50* and *lig4* mutants and their isogenic and untransformed wild-type control (C). (**B**) T-DNA transformed *mre11*, *xrs2* and *sir4* mutants and their isogenic and untransformed wild-type control (C). White arrowheads indicate chromosomes with an altered mobility.

observation may be explained by the idea that Rad52 and Yku70 are competing agents, channelling recombination into either HR or NHR, respectively (Haber, 1999). Yku70-mediated integration of T-DNA by NHR may be more efficient as the competing pathway of HR is not operative in the absence of Rad52.

T-DNA integrates preferentially at (sub)telomeric regions in *rad50*, *mre11* and *xrs2* mutants. The telomeres comprise ~2% of the yeast genome (Zakian, 1996). Therefore, 2% of the T-DNA insertions would be expected at the telomeres. In fact in the wild-type we have found one telomeric T-DNA insertion in 54 analysed wild-type lines (Table II). In contrast, in the *rad50*, *xrs2* and *mre11* mutants eight out of 11 T-DNAs had integrated in this area. The Rad50, Mre11 and Xrs2 proteins play a minor role in telomeric silencing (Boulton and Jackson, 1998). Thus, an explanation might be that reduced silencing at the telomeric region makes this part of the chromosome more accessible for T-DNA, thereby facilitating T-DNA integration at (sub)telomeric regions. The Sir4 protein plays an important role in transcriptional silencing at the telomeres (Aparicio *et al*., 1991). However, we did not find a bias for T-DNA integration at these regions in the *sir4* mutant. Therefore, we conclude that the absence of telomeric silencing in the *rad50*, *mre11* and *xrs2* mutants is not responsible for the integration of T-DNA at the telomeres. We speculate that an alternative T-DNA integration pathway, which leads to specific integration at the telomeres or ribosomal DNA repeat, can replace the normal integration pathway in the absence of an active Rad50–Mre11–Xrs2 complex. One possibility is that this pathway may operate *in vivo* to repair DNA aberrations occurring during DNA replication of repeated DNA. Alternatively, it may be used for the restoration of the telomeric structure. The Rad50–Mre11–Xrs2 complex and the Yku70 protein are involved in telomere length

Table III. Yeast strains

Strain	Genotype	Reference
YPH250	*MATα, ura3-52, lys2–801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1*	Sikorski and Hieter (1989)
YPH250*rad51*	*MATα, ura3-52, lys2–801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, rad51::LEU2*	this study
YPH250*rad52*	*MATα, ura3-52, lys2–801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, rad52::LEU2*	this study
YPH250*yku70*	*MATα, ura3-52, lys2–801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, yku70::LEU2*	this study
YPH250*rad50*	*MATα, ura3-52, lys2–801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, rad50::hisG*	this study
YPH250*lig4*	*MATα, ura3-52, lys2–801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, lig4::HIS3*	this study
JKM115	*MATα, Δho, Δhml::ADE1, Δhmr::ADE1, ade1, leu2-3,112, lys5, trp1::hisG, ura3-52*	Moore and Haber (1996)
JKM129	*MATα, Δho, Δhml::ADE1, Δhmr::ADE1, ade1, leu2-3,112, lys5, trp1::hisG, ura3-52, xrs2::LEU2*	Moore and Haber (1996)
JKM138	*MATα, Δho, Δhml::ADE1Δhmr::ADE1, ade1, leu2-3,112, lys5, trp1::hisG, ura3-52, mre11::hisG*	Moore and Haber (1996)
JKM120*sir4*	*MATα, Δho, HMLα, HMRα, ade1, leu2-3,112, lys5, trp1::hisG, ura3-52, his3::hisG′-URA3-hisG′, sir4::HIS3*	Lee *et al*. (1999)

maintenance and *rad50*, *mre11*, *xrs2* and *yku70* mutants show shortened telomeres (Porter *et al*., 1996; Boulton and Jackson, 1998). The telomeric structure has to be restored in order to survive and this process may lead to the incorporation of T-DNA at the telomeres. As we did not obtain such T-DNA insertions in the *yku70* mutant, it may be that *YKU70* plays an important role in this pathway.

It has been found that the lack of *Sir* genes induces the loss of silencing of cryptic mating-type genes. This leads to changes in expression of mating-type specific genes, which in turn leads to a reduced efficiency of NHEJ (Lee *et al*., 1999). The Sir4 protein may also play a minor role in DSB repair by NHEJ by mediating an altered local chromatin structure at the break site, thereby making the break site more accessible for the repair enzymes (Boulton and Jackson, 1998; Critchlow and Jackson, 1998). Both options can explain why the T-DNA integration frequency is reduced in the *sir4* mutant and the pattern of integration remains as in the wild-type.

In the absence of Rad50, Mre11 and Xrs2, and to a lesser extent Lig4, gross chromosomal rearrangements have been observed (Chen and Kolodner, 1999). In our studies we did not detect such chromosomal rearrangements, neither in untransformed wild-type nor in the untransformed *rad50*, *mre11*, *xrs2* and *lig4* mutants (data not shown). However, chromosomal rearrangements were often seen in the yeast mutants after transformation by *Agrobacterium* and more specifically in the *rad50*, *mre11* and *xrs2* mutants after the T-DNA had integrated at (sub)telomeric regions. We infer that either the integration of T-DNA or the presence of a T-DNA insertion at (sub)telomeric regions is specifically accompanied by chromosomal rearrangements. It has been observed before that Ty elements can induce chromosomal rearrangements by inserting at telomeric regions (Kim *et al*., 1998). Alternatively, T-DNAs may be captured specifically by cells with chromosomal instability. Translocations were the principal class of rearrangements seen in *rad50*, *mre11* and *xrs2* mutants (Myung *et al*., 2001) and might reflect the rearrangements seen in the *rad50*, *mre11* and *xrs2* mutants in which the T-DNA had integrated at the (sub)telomeric region. However, further experiments should elucidate the type of rearrangements observed in these mutants.

We observed that in the absence of Lig4 T-DNA integration is strongly reduced. It was remarkable that the few lines that were obtained after transformation by *Agrobacterium* had T-DNA repeat structures. We may assume that due to the absence of Lig4 chromosomal breaks are sealed less efficiently and that the T-DNAs that are captured by such breaks have more time to recombine or fuse to extrachromosomal copies of T-DNA, resulting in the accumulation of T-DNAs in repeat structures. The repeats consist of fused left and right borders and it is therefore likely that the VirD2 strand transferase mediated formation of these repeat structures (Pansegrau *et al*., 1993).

The structure of the T-DNA as seen after integration by NHR into the genome of plants, fungi and yeasts is similar (Mayerhofer *et al*., 1991; Bundock and Hooykaas, 1996; De Groot *et al*., 1998). This has been taken as evidence that T-DNA integration in these diverse organisms occurs by a conserved mechanism. In agreement with this expectation the NHR machinery, which we found to be used for random T-DNA integration in yeast, has been well conserved during eukaryotic evolution. Further experimentation will have to prove that plant and fungal orthologues of the yeast *YKU70*, *RAD50*, *MRE11*, *XRS2*, *LIG4* and *SIR4* genes also play an important role in T-DNA integration in these organisms.

Materials and methods

Yeast strains

The yeast strains that were used are listed in Table III. Yeast mutants isogenic to the haploid YPH250 strain were constructed using the one-step disruption method (Rothstein, 1991) after lithium acetate transformation (Gietz *et al*., 1992). Disruption of *YKU70*, *LIG4*, *RAD50*, *RAD51* and *RAD52* was confirmed by PCR and Southern blot analysis.

Construction of the binary vector pSD8000

To construct pSDM8000, a 1513 bp *Pvu*II–*Eco*RV fragment carrying the *KanMX* marker was obtained from pFA6a (Wach *et al*., 1994) and was ligated into the unique *Hpa*I site of pSDM14 (Offringa, 1992). The binary vector pSDM8000 was introduced into *A.tumefaciens* by electroporation (Den Dulk-Ras and Hooykaas, 1995).

Co-cultivations/T-DNA transfer experiments

Co-cultivations were performed as described earlier with slight modifications (Bundock *et al*., 1995). *Agrobacterium* strain LBA1119 (also known as EHA105; Hood *et al*., 1993) was used in all experiments and was grown overnight in LC medium prior to co-cultivation. The mix of *Agrobacterium* and *S.cerevisiae* cells was incubated for 9 days at 20°C. T-DNA containing *S.cerevisiae* strains were selected at 30°C on YPAD medium containing G418 (200 μg/ml) (Life Technologies/Gibco-BRL).

Vectorette PCR and TAIL–PCR

Chromosomal DNA was isolated using Qiagen's Genomic Tips G/20 as per manufacturer's protocol. An amount (1–2 μg) of genomic DNA was

digested with *Eco*RI, *Cla*I, *Pst*I or *Hin*dIII and run on a 1% TBE gel. Non-radioactive Southern analysis was performed. The membrane was hybridized with a digoxigenine-labelled KanMX probe to determine the size of T-DNA/genomic junction fragments (*Eco*RI and *Cla*I for right end containing fragments and *Pst*I and *Hin*dIII for left end containing fragments). The KanMX probe, a 792 bp internal fragment of the *KanMX* marker, was made by PCR using primers kanmxp1 (5′-AGACT-CACGTTTCGAGGCC-3′) and kanmxp2 (5′-TCACCGAGGCAGTTC-CATAG-3′) and a Non-Radioactive DNA Labelling and Detection kit (Boehringer Mannheim). The restriction enzyme producing the smallest junction fragment was used for Vectorette PCR as described (http://genome-www.stanford.edu/group/botlab/protocols/vectorette.html). The Expand™ High Fidelity System (Boehringer Mannheim) was used to amplify fragments >2.5 kb, whereas sTaq DNA polymerase (SphaeroQ) was used for the amplification of fragments <2.5 kb. Primer kanmxp3 (5′-TCGCAGGTCTGCAGCGAGGAGC-3′) and kanmxp4 (5′-TCGCT-CGACATCATCTGCCCAG-3′) were used to amplify right border junction and left border junction fragments, respectively.

TAIL–PCR was used to isolate right border junction fragments and was performed as described previously (Liu *et al*., 1995), except that KanMXp3, KanMXp5 (5′-TCACATCATGCCCCTGAGCTGC-3′) and KanMXp7 (5′-GGGTATTCTGGGCCTCCATG-3′) were used as right border specific primers.

The yeast genomic sequences that were isolated by Vectorette and TAIL–PCR were used in a BLAST search in the yeast genome database at http://www-genome.stanford.edu/SGD in order to determine the T-DNA integration sites.

T7 DNA polymerase sequencing

Vectorette PCR and TAIL–PCR products were cloned in pGEMTEasy (Promega) and sequenced using the T7 polymerase sequencing kit (Pharmacia) according to the manufacturer's protocol.

CHEF gels

Complete and intact yeast chromosomes were isolated in agarose blocks as described previously (Schwartz and Cantor, 1984; De Jonge *et al*., 1986). Blocks were placed in the wells of a 0.25× TBE–agarose gel. A CHEF apparatus was used to separate the chromosomes (Bio-Rad). Electrophoresis was performed in 0.25× TBE at 14°C with an initial switch time of 40 s and a final switch time of 120 s at 200 V for 30 h. The LKB2016 VacuGene vacuum blotting apparatus was used with 10× standard saline citrate (SSC) to transfer the chromosomes to a positively charged Nylon membrane (Boehringer Mannheim). The membrane was probed with the KanMX probe (see Vectorette PCR) and autoradiography was done for 1 day using Fuji SuperRX film. Subsequently, the membrane was stripped in 0.5 M NaOH at 42°C, hybridized with a probe specific for chromosome XII, stripped again and hybridized with a probe specific for chromosome VII. Probes specific for chromosomes XII and VII, were made by PCR. A 1045 bp region, located downstream of the *RAD5* gene, and a 1246 bp internal fragment of the *LIF1* gene was amplified from genomic DNA isolated from the wild-type strain YPH250, respectively, using primers Lif1p1 (5′-AGCTGACGGAGTTCATTAGCT-3′) and Lif1p2 (5′-TACCGTTTCCGATTCTGTCT-3′) and Rad5p5 (5′-GGATTCGACAACAAGGGGTC-3′) and Rad5p6 (5′-GTGTGGTAA-GAGCACCTGCC-3′).

Acknowledgements

We thank Professor J.Haber (Rosenstiel Center and Biology Department, Brandeis University, Waltham, MA) for kindly providing a number of yeast mutants, Dr H.Y.Steensma for technical assistance with the CHEF gel and Dr E.J.van der Zaal and Dr R.Offringa for critical reading of the manuscript. This work was supported by the EU RECOMBIO Biotech program BIO4-CT97-2028.

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Received July 13, 2001; revised September 6, 2001; accepted September 19, 2001